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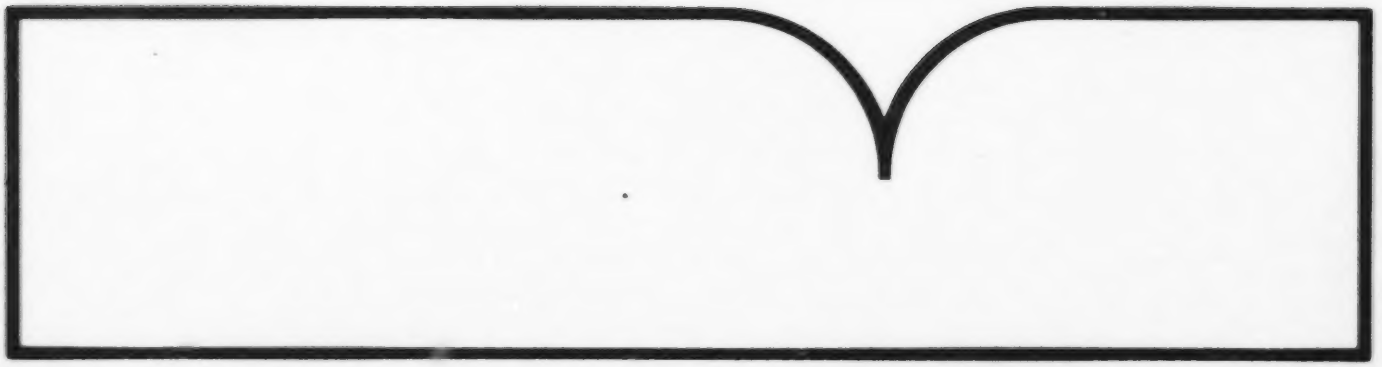

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BIOLOGICAL DETOXIFICATION RESEARCH:

POTENTIAL USES FOR A FUNGAL SPECIES

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Starting in 1984, the Hazardous Waste Engineering Research Laboratory of the U.S. Environmental Protection Agency has funded research to determine the feasibility of using P. chrysosporium to degrade organic pollutants.

The white rot fungus research continues to stimulate interest and shows promise for field applications. The parts of the research program sponsored by the Hazardous Waste Engineering Research Laboratory are starting to develop major advances in the emerging biological control technology field.

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ABSTRACT

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INTRODUCTION

White rot fungi are primary wood degraders in nature (1). All components of wood are degraded by these fungi including lignin which is a structural component of wood composed of a heteropolymer formed from the cross linking of three precursor cinnamyl alcohols (2).

The structural features of lignin dictate unusual constraints on the biodegradation systems responsible for the initial attack on lignin. The 600-1000 k-dalton size range for lignin is far too large to enter cells. The requirements are that the degradative system must be extracellular, non-specific, and non-hydrolytic. Analogies with other biopolymers degrading systems fail since these other systems are hydrolytic and specific (2).

Some of the more common substructures of lignin, catechol diethers, alkylarenes, and biphenyls, resemble the chemical structure of many persistent organic compounds contaminating the environment. The remarkable similarity in structure led several investigators to research the applicability of a white rot fungus, Phanerochaete chrysosporium, to the biodegradation of hazardous waste constituents (2).

Since the findings of Aust (3) and Eaton (4), the area of application of the white rot fungus, P. chrysosporium, to the detoxification of organic compounds designated as hazardous waste constituents has progressed significantly. Starting in 1984, the Hazardous Waste Engineering Research Laboratory of the U.S. Environmental Protection Agency has funded research to determine the feasibility of using P. chrysosporium to degrade organic pollutants.

Data on degradation by white rot fungus have resulted from the Michigan State University research team's efforts (now at Utah State University) and more recently the new experimental approaches of the USDA Forest Products Laboratory and the State University of New York at Syracuse. This research sponsored by the USEPA represents a structured and targeted program of individual projects focusing on the short term development of this microorganism for application in a field setting for treatment of contaminated soil and water.

COMPETENCE AND NUTRITIONAL STUDIES (5)

In the first report of this work, Bumpus et al. (3) showed that the fungus oxidized 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT), 3,4,3',4'-tetrachlorobiphenyl, 2,4,5,2',4',5'-hexachlorobiphenyl, 2,3,7,8-tetrachlorodibenzo[p]dioxin (TCDD), lindane, and benzo(a)pyrene to carbon dioxide. Subsequent investigations showed that the fungus is capable of mineralizing hexachlorobenzene, Mirex, Aroclor 1242 (6), Aroclor 1254 (4, 7), chlordane, pentachlorophenol, p-cresol, phenanthrene, 2-methylnaphthalene, and biphenyl (7), benzoic acid (8), and Atrazine, naphthalene, and methoxychlor (9).

DDT was used as a model compound for detailed biodegradation experiments (10). In this work determinations were made on DDT disappearance, metabolite identification, mass balance analysis, and ^{14}C-DDT mineralization. It was found that P. chrysosporium causes extensive degradation of DDT in nutrient nitrogen-deficient cultures. The metabolites from DDT that were identified included 2,2,2-trichloro-1,1-bis(4-chlorophenyl)ethanol (dicofol), 1,1-dichloro-2,2-bis(4-chlorophenyl)ethane (DDD), 2,2-dichloro-1,1-bis(4-chlorophenyl)ethanol (FW-152), and 4,4'-dichlorobenzophenone (DBP). These results allowed for the determination of a proposed pathway different from the major pathway proposed for bacterial degradation of DDT occurring in the environment. In this work it was determined that other fungi, Pleurotus ostreatus, Phellinus weirii, and Polyporus versicolor, also mineralized DDT.

It was found that with glucose as a growth substrate the rate and extent of DDT mineralization increased with increasing glucose concentration (11, 12). This held true for the glucose test concentrations between 23 mM and 112 mM. The greatest initial rate of mineralization, however, occurred when the organism was grown on 23 mM glucose. Also, as the concentration of glucose became limiting the rate of mineralization quickly declined.

Alternative growth substrates differed in their effects upon DDT mineralization (9). Glucose, fructose, mannose, mannitol, and glycerol supported growth and mineralized DDT while polyethylene glycol, glycine, and benzoic acid did not support growth of the fungus or mineralization of DDT. Cellulose,

a growth substrate for the fungus in nature, under experimental conditions increased the amount of DDT mineralization approximately two fold relative to glucose. The authors suggested that more efficient growth and mineralization of organic pollutants may be possible when the fungus is grown on cellulose because of the organism's ability to regulate the availability and utilization of glucose.

Hydrogen peroxide, a required cofactor/activator of ligninases, can be produced by a number of carbohydrate oxidases and other enzymes in wood rotting fungi. It is known that a major source of hydrogen peroxide is the glucose oxidase system when glucose is the growth substrate (9). It is believed then that mineralization of organic pollutants is dependent upon the ability of the fungus to generate H_2O_2. Ongoing research is attempting to determine the specifics of the action of H_2O_2 on growth and degradation.

ENZYME STUDIES (13)

Among the more important reactions in lignin breakdown by P. chrysosporium are cleavage of the lignin alkyl side chain, ring demethylation, and ring cleavage. The alkyl side chain cleavage is catalyzed by a hemoprotein ligninase discovered in 1983. Hydrogen peroxide is consumed in this reaction with corresponding changes in the enzyme absorption spectrum during catalysis attributable to resting states-compound I-compound II spectral transients indicating a peroxidative mechanism (12). Stoichiometries of product formation as well as hydrogen peroxide and oxygen uptake are consistent with a radical pathway (14). These results established the one-electron oxidative mechanism as the primary extracellular oxidative pathway for P. chrysosporium.

It has been shown that P. chrysosporium produces at least ten extracellular hemoproteins and roughly half have ligninase activity (assayed by 3,4-dimethoxybenzyl alcohol oxidation) (15). The enzyme component designated H8 has been used by several researchers to characterize the ligninase activity. Depending on growing conditions, H8 can be displaced as the major component in favor of H2 (16). The separation of the extracellular enzymes into fast protein liquid chromatography (FPLC) resolvable bands does not indicate true homogeneity within each band since some of the bands can be separated into subcomponents by electrophoresis. The extracellular hemoproteins have distinct amino acid sequences hence they are separate gene products and not merely degradation products of a single precursor. The heme components H3-H5 have manganese peroxidase activity. This three component fraction catalyzes a hydrogen peroxide-dependent oxidation of Mn(II) to Mn(III) but lacks the specificity of H8 to cleave the alkyl side chains. Two reports (17, 18) of soluble manganese ion acceleration on this fraction prompted further inspection of the enzymatic activity of these peroxidases. The current status of this research is unclear with respect to the reported accelerations. Spectral contamination has been observed that contributes to a complex situation for rate data interpretation. Work is continuing to clarify this situation.

The heterogeneity among the various extracellular proteins produced by P. chrysosporium points to possible functional differences among them which are important to pollutant degradation. There presently are attempts to uncover substrate specificities where information indicates that they may exist.

The ability of the ligninase H8 to oxidize polynuclear hydrocarbons has been related to the ionization potential of these compounds. When pyrene is used as a substrate with H8 both pyrene-1,6-dione and pyrene-1,8-dione are the major products. In similar fashion, anthracene is converted to anthraquinone and benz(a)anthracene yields 7,12-benz(a)anthraquinone (19). Both the pyrene-1,6-dione and pyrene-1,8-dione are mutagenic by the Ames test. When the compounds are presented as substrates to the fungus these diones do not accumulate.

Dibenzodioxin and 2-chlorodibenzodioxin are oxidized by the H8 ligninase in the presence of hydrogen peroxide (19). Only determination of radical cation intermediates by flow cell ESR studies was made for these compounds without any product identification. Current work is directed to determine whether chlorinated aromatics are substrates (20, 21). Chlorinated phenols have been found to be very suitable substrates, and product identification is under way (22).

SOIL STUDIES (23, 24)

The general success of solution biodegradation studies with the fungus stimulated speculation that this microorganism may be an appropriate candidate for the treatment of contaminated soils. A research program was initiated with the USDA Forest Products Laboratory designed to specifically investigate and develop a soil treatment system based on the white rot fungus. Research to date has assessed the effects of selected soil types, temperatures, pH, and water potentials on the growth of the fungus in sterile and non-sterile soils. Biomass accumulations as well as growth habits of P. chrysosporium were greatly influenced by soil type. Soil nitrogen content appears to be the primary factor responsible for differences in fungal growth in three studied soils. Growth was strongly and positively correlated with nitrogen content. This factor, therefore, appears to play a major role in mediating the growth of the fungus in the soil, and is easily controlled by nitrogen supplementation.

Increasing the soil water potential from -1.5 MPa to -0.03 MPa resulted in greatly increased growth of P. chrysosporium. Research data suggest that fungal growth might benefit from soil water potentials greater than -0.03 MPa. Water potential is an easily controlled soil factor in field scale demonstration conditions.

Early work in this investigation indicated that P. chrysosporium did not grow well in non-sterile soils attributable in part due to ineffective competition with the indigenous microflora. Lately it has been found that growth within the soil can be accomplished through the use of larger quantities of inoculum. These results were anticipated since the soil is not the normal habitat of P. chrysosporium.

The white rot fungus grows over a wide range of temperatures. Growth has been assessed from 10 to 39°C. No growth was observed at 10°C whereas growth significantly increases with temperature from 15 to 30°C. No significant difference in growth was recorded from 30 to 39°C. Soil temperatures under field conditions can be controlled by selecting the normal warm months and by soil solarization.

Future investigations in the soil application research will include small scale treatment of selected pollutants at environmentally significant concentrations, the evaluation of growth stimulating amendments on primary and secondary metabolism, and the delivery of oxygen to the growing fungus within the soil column. Ancillary investigations will include the development of analytical procedures to assay the fungal growth within the soil, the importance of soil sterilization to growth of the fungus, and inoculum development.

TECHNOLOGY DEVELOPMENT

A water treatment process developed jointly by North Carolina State University and the USDA Forest Products Laboratory will be investigated in the next year at the bench and scaled up to determine its treatment efficacy. The patented reactor (25) is a specially designed rotating biological contactor that utilizes P. chrysosporium as the biological species for treatment. Initial success with color removal for kraft chlorine bleaching plant effluent has led to more detailed studies (26, 27). Numerous chlorinated organics produced during the chlorine bleaching of wood pulp were shown to be degraded by the white rot fungus in this RBC reactor (28). Recent results derived for the operation of this technology show that this reactor will degrade 250 ppm pentachlorophenol in water to 5 ppm in 8 hours (29). Pink water associated with munitions production is adequately treated. Degradation of 2,4,6-trinitrotoluene and 2,4-dinitrotoluene is observed in concentrations up to 150 ppm in 24 hours (29).

CONCLUSIONS

White rot fungus research continues to stimulate interest and shows promise for field applications. The parts of the research program sponsored by the Hazardous Waste Engineering Research Laboratory are starting to develop major advances in the emerging biological control technology field.

Additional research will be pursued to increase the potential for white rot fungus as a practical degrader of hazardous pollutants. Possible programs for consideration as future research areas are the selection for strains that are high producers of ligninases, selection for strains capable of producing ligninases regardless of the nutrient starvation requirement, and optimizing culture conditions for ligninase production. Enzyme production is expected to be improved by means of gene cloning techniques. The cDNA for a ligninase

from P. chrysosporium has already been cloned and sequenced (30, 31). This accomplishment shortens the time it will take to develop, by means of biotechnology techniques, super producers of the white rot fungus enzymes that are so effective as pollutant degraders.

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